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14. ABSTRACT
Low levels of dietary selenium are associated with an increased incidence of a number of malignancies, including liver, lung, prostate, colon and pancreas cancers. Additionally, specific selenium compounds such as selenomethionine have a direct growth inhibitory action on numerous types of cancer cells, including breast cancer cell lines [1]. It has also been shown that selenium displays a potent anti-cancer effect against experimentally-induced mammary cancers in rodents. It has been proposed that a 56 kDa "selenium binding protein" (SBP-56; SP56) that was found to be abnormal or absent in a high percentage of experimental mammary carcinomas in rodents, plays an important role in mediating the chemopreventive action of selenium [2]. Additionally, our laboratory has found that the homologous human SP56 (hSP56) [3] displays differential expression between normal and neoplastic human tissues and between androgen-sensitive LNCaP and androgen-insensitive PC-3 prostate cancer cell lines, which suggests a role for hSP56 in determining the neoplastic phenotype and in mediating the cancer-protective action of dietary selenium [4]. Indeed, hSP56 has been associated with the growth inhibitory action of selenomethionine in human prostate cancer cells [5]. Since hSP56 has not been studied in human breast cancer yet, it is possible that it mediates selenium-modulated growth inhibition in human breast cancer cells as well, which may open the way for development of new preventive and/or therapeutic modalities.

15. SUBJECT TERMS
cell biology, selenium, proliferation, chemoprevention

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INTRODUCTION

It is our hypothesis that human selenium binding protein hSP56 plays important and yet unrecognized roles in the regulation of cell proliferation, and/or apoptosis, or other processes in normal and malignant mammary cells in response to environmental changes and selenium availability. The aim of our study is to determine if hSP56 can mediate selenium growth inhibition of human breast cancer cells. This will broaden our understanding of the role of hSP56 in cancer development and progression with regard to the potent anti-cancer action of selenium compounds. Identification of hSP56 as a mediator of selenium growth-inhibitory action in human breast cancer cells will contribute to the elucidation of the mechanisms of selenium chemopreventive action in general and could lead to the development of selenium-containing small molecules as preventive or therapeutic drugs.

BODY

We started our study by selecting a number of human breast cancer cell lines that differ in their invasiveness *in vitro* and tumorigenicity *in vivo* (Table 1). MCF-7 and T-47D cell lines are representative of weakly tumorigenic estrogen-responsive breast cancer cells, while the SK-BR-3 cells do not respond to estrogen and are tumorigenic *in vivo*. The MDA-MB-231 and Hs578T cell lines are representative of poorly differentiated, highly invasive mammary carcinomas. They do not express any of the epithelial markers found in MCF-7, T-47D and SK-BR-3 cell lines and they are highly invasive *in vitro*. MDA-MB-231 cells are also highly tumorigenic and highly metastatic *in vivo*, while Hs578T cells are not tumorigenic in immunosuppressed mice, but they do form colonies in semisolid medium. We also obtained two non-tumorigenic breast cell lines. The Hs578Bst cell line is the “normal” fibroblast-like counterpart of Hs578T cells and was established from normal tissue peripheral to the infiltrating ductal carcinoma which was the source of the cancer cell line. The MCF-10A cell line is an immortalized non-tumorigenic mammary epithelial line. Although MCF-10A cells are not tumorigenic in immunosuppressed mice, they are able to form colonies in semisolid medium.

Table 1. **Characteristics of breast cancer cell lines used in this study.**

Cell line	**Tumorigenicity *in vivo***	**Invasiveness *in vitro***	**Phenotype/ genotype**	**Morphology on plastic**	**ER/PgR**	**other**
MCF-7	poorly (estrogen dependent)	weakly	luminal epithelial-like	polygonal	+/+	typical epithelial markers
T-47D	poorly (estrogen dependent)	weakly	luminal epithelial-like	polygonal	+/+	typical epithelial markers
SK-BR-3	yes	weakly	weakly luminal epithelial-like	clusters of weakly attached cells	-/-	amplified *ERBB2*
MDA-MB-231	highly	highly	stromal/ mesenchemal	fibroblastoid	-/-	over-express vimentin
Hs578T	yes (semisolid medium)	highly	stromal/ mesenchemal	fibroblastoid	-/-	over-express vimentin

The level of hSP56 expression in five breast cancer cell lines (MCF-7, SK-BR-3, T-47D, MDA-MB-231, Hs578T), and two immortalized non-tumorigenic, non-metastatic mammary epithelial cell lines (MCF-10A and Hs578Bst) was determined by western blot and RT-PCR. The results are summarized in Table 2.

Our western blot analyses showed that hSP56 protein is present at very low levels in the non-tumorigenic MCF-10A cell line (Fig. 1). On the other hand, we found that hSP56 is expressed at high levels in the weakly invasive breast cancer cell lines (MCF-7, SK-BR-3 and T-47D; Figs. 1 and 2) and at undetectable levels in the highly invasive and tumorigenic MDA-MB-231 (Fig. 2) and Hs578T (Fig. 3) cells. We did not detect hSP56 protein in the immortalized breast cell line Hs578Bst.

Table 2. **Relative protein expression levels of hSP56 in cell lines used in this study determined by western blot.**

Cell line	hSP56 expression level
LNCaP	+++++
MCF-7	++++
SK-BR-3	++++
T-47D	++
MCF-10A	+
MDA-MB-231	-*
Hs578T	-*
Hs578Bst	-
PC-3	-*

*RT-PCR analysis (see below) revealed the presence of hSP56 mRNA in MDA-MB-231 and Hs578T RNA and its absence in the case of PC-3 cells, a negative control cell line. RT-PCR analysis on Hs578Bst cells was not performed.

Our RT-PCR studies confirmed the presence of hSP56 mRNA transcripts in MCF-10A, SK-BR-3, MCF-7, T-47D, Hs578T and MDA-MB-231 (Figs. 4-6). Although hSP56 protein could not be detected by western blot in MDA-MB-231 and Hs578T cells, RT-PCR analysis revealed that its mRNA transcript is present in these cells.

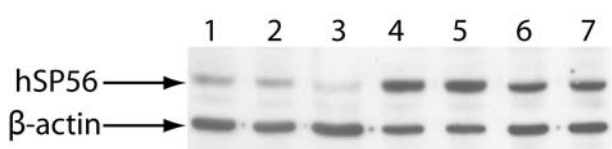

Figure 1. **Detection of hSP56 protein expression in MCF-10A and MCF-7 cells by Western blot.** Western blot analysis shows high level of expression of hSP56 in MCF-7 breast cancer cells (lanes 6 and 7) and low amount of hSP56 in non-tumorigenic MCF-10A breast cells (lanes 1 and 2). LNCaP prostate cancer cells (Lanes 4 and 5) express high levels of hSP56 and were used as positive control, while PC-3 prostate cancer cells (lane 3) that express very low amounts of hSP56 were used as a negative control. Immunoblotting with antibody against β-actin served as a loading control.

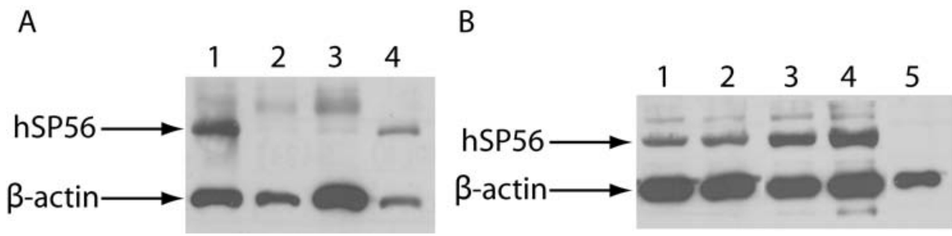

Figure 2. **Western blot analysis of hSP56 protein expression in MDA-MB-231, T-47D and SK-BR-3 breast cancer cells.** A) hSP56 expression was not detected in the highly invasive breast cancer cell line MDA-MB-231 (lanes 2 and 3). hSP56 expressed in MCF-7 breast cancer cells (lanes 1 and 4) was used as a positive control. B) hSP56 is present in higher levels in SK-BR-3 (lanes 3 and 4) than T-47D cells (lanes 1 and 2). Here MDA-MB-231 cell lysates were used as a negative control (lane 5). Immunoblotting with antibody against β-actin served as a loading control.

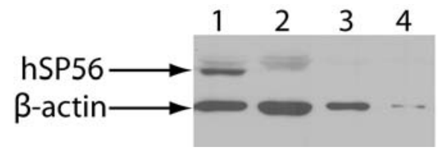

Figure 3. **Western blot analysis of hSP56 protein expression in Hs578T, Hs578BSt and Hs578T/hSP56 cells**. hSP56 was not detected in both the highly invasive breast cancer cell line Hs578T (lane 2) and in the non-tumorigenic Hs578Bst cell line (lanes 3 and 4). hSP56 expressed in Hs578T cells transiently transfected with a mammalian expression vector containing hSP56 cDNA (lane 1). Immunoblotting with antibody against β-actin served as a loading control.

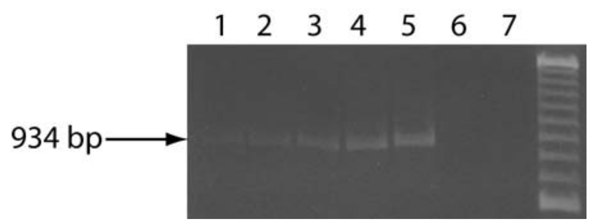

Figure 4. **hSP56 is expressed in MCF-10A and SK-BR-3 cells.** RT-PCR analysis of total RNA isolated from non-tumorigenic immortalized MCF-10A breast cells (lanes 1 and 2) and SK-BR-3 breast cancer cells (lanes 4 and 5) reveals the presence of hSP56 mRNA transcript. LNCaP (lane 3) prostate cancer cells were used as positive control. The minus cDNA control (lane 6) and the minus reverse transcriptase control (lane 7) confirm the absence of DNA contamination.

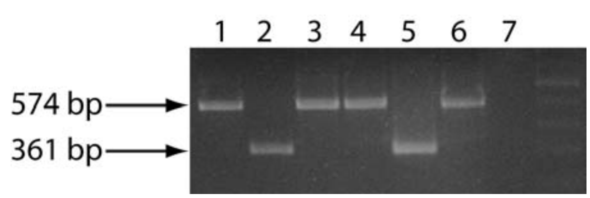

Figure 5. **hSP56 is expressed in T-47D and MCF-7 cells.** RT-PCR analysis confirms that hSP56 mRNA (574 bp fragment) is present in T-47D (lanes 1 and 3) and MCF-7 breast cancer cells (lanes 4 and 6). A 361 bp fragment from a highly conserved region of a constitutively expressed "housekeeping" gene was amplified with control primers as a positive control for each cell line (lanes 2 and 5). The minus reverse transcriptase control (lane 7) confirms the absence of DNA contamination.

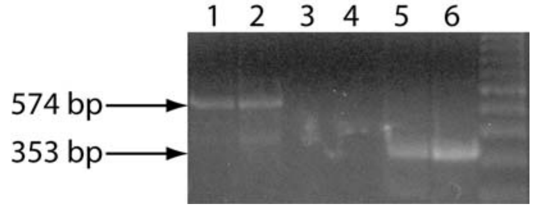

Figure 6. **hSP56 mRNA was detected in Hs578T and MDA-MB-231 cells.** RT-PCR analysis shows that hSP56 mRNA (574 bp fragment) is transcribed in Hs578T (lane 1) and MDA-MB-231 (lane 2) breast cancer cells. A 353 bp fragment from a highly conserved region of β-Actin was amplified as a positive control for each cell line (lanes 5 and 6). The minus reverse transcriptase controls (lanes 3 and 4) confirm the absence of DNA contamination in RNA preparations from each cell line.

It was shown recently that selenomethionine (SeMet) inhibits the growth of LNCaP, but not PC-3 prostate cancer cells and it was proposed that this difference might be due to the fact that LNCaP cells express hSP56, whereas PC-3 cells do not [5]. Indeed, our recent gain-of-function and loss-of-function studies on LNCaP and PC-3 cells strongly support the hypothesis that hSP56 plays a critical role in mediating the growth-inhibiting action of SeMet on prostate cancer cells. In this study we wanted to determine how different breast cancer cell lines with different hSP56 expression profiles respond to SeMet treatment.

To determine the growth rates of the selected breast cancer cell lines *in vitro* we used the MTT cell proliferation assay which offers a quantitative measure of the number of viable cells in a population. Cells were seeded in replicates in 96-well plates at 3000 cells/well and 24 h after seeding they were treated with various concentrations of SeMet. After 0, 24, 48 and 72 hours of treatment the MTT assay was performed. The absorbance measured at 570 nm was directly proportional to the number of viable cells, which was confirmed in separate experiments using trypan blue cell counting.

Using this methodology we showed that SeMet inhibits the growth of the weakly invasive estrogen-responsive MCF-7 (Fig. 7) and T-47D (Fig. 8) breast cancer cells, which express high levels of hSP56 protein, in a time- and concentration-dependent manner. However, SK-BR-3 cells (Fig. 8), despite the presence of hSP56, were resistant to the growth inhibitory actions of SeMet. Factors other than the presence or absence of hSP56 may govern the resistance of the estrogen-independent SK-BR-3 cells to SeMet. On the other hand, SeMet did not show a potent inhibition of growth of the highly tumorigenic MDA-MB-231 and Hs578T breast cancer cells (Fig. 9) which have undetectable levels of hSP56 protein. The fact that the effect of SeMet was much more pronounced in the case of MCF-7 and T-47D cells than MDA-MB-231 and Hs578T cells suggests that hSP56 may have an important role in mediating the growth-inhibitory action of SeMet in human breast cancer cells.

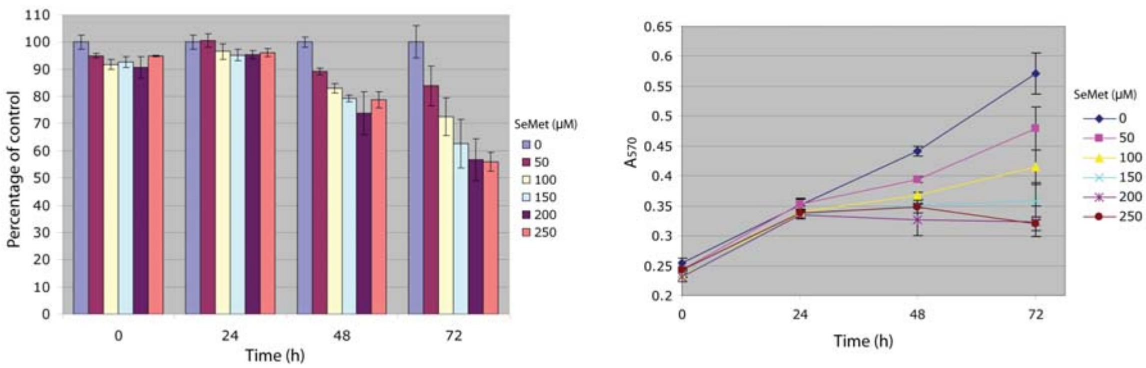

Figure 7. **Selenomethionine inhibits the growth of MCF-7 cells in time- and concentration-dependent manner.** MCF-7 cells were grown in triplicates in the presence of increasing concentrations of SeMet for 3 days and the MTT cell proliferation assay was performed on a daily basis. The absorbance measured at 570 nm (presented with the growth curves on the right) is directly proportional to the number of viable cells. The graph on the left hand side shows that by the third day the number of MCF-7 cells grown in the presence of 250 µM SeMet decreased to about 50% in comparison with the control treated with vehicle only.

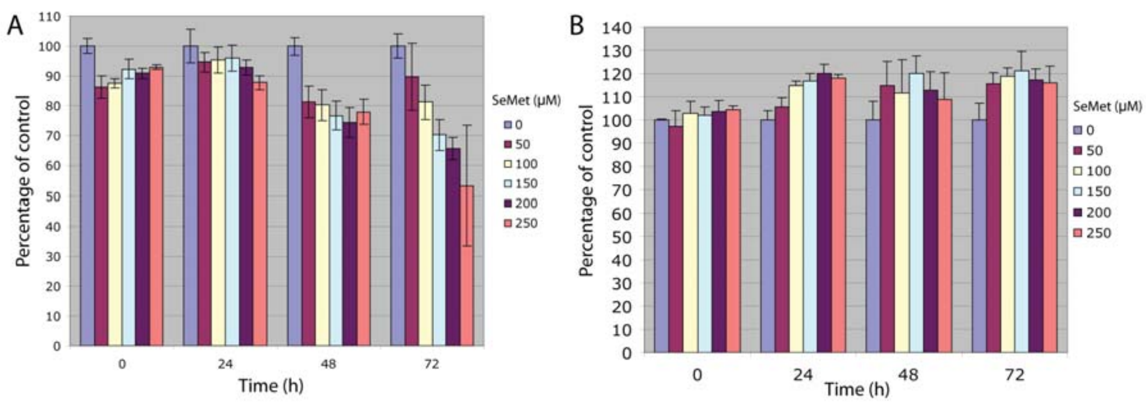

Figure 8. **Selenomethionine inhibits the growth of T-47D cells, but not SK-BR-3 cells.** The number of T-47D cells (A) treated with 250 µM SeMet for 72 h decreased to about 50% in comparison with the control cells treated with vehicle only. On the other hand, SeMet did not inhibit the growth of SK-BR-3 cells (B), which even showed an increase in their number over the whole range of SeMet concentrations and time of treatment.

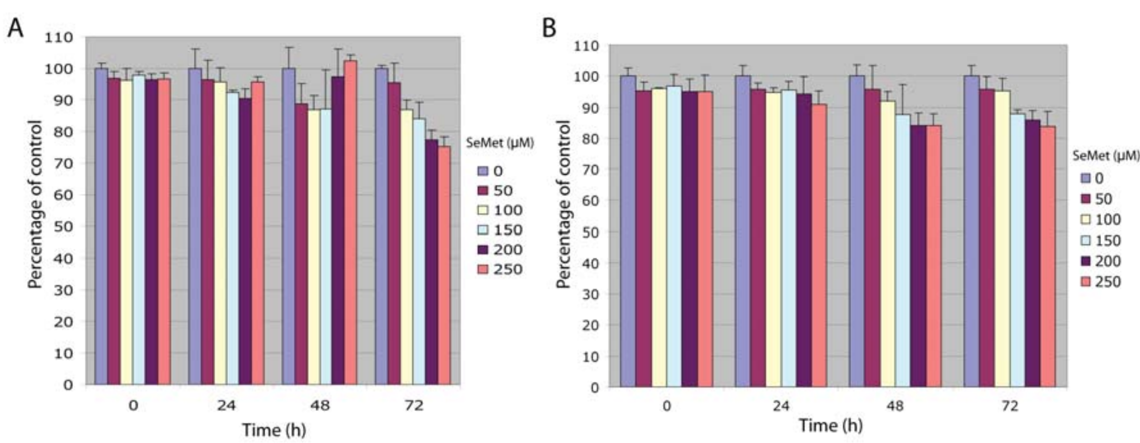

Figure 9. **Selenomethionine does not show a potent inhibition of growth of MDA-MB-231 and Hs578T cells.** The number of MDA-MB-231 (A) and Hs578T (B) cells, which do not express a detectable amount of hSP56, treated with 250 µM SeMet for 72 h decreased to about 75% and 85%, respectively.

In order to confirm this, we performed gain-of-function studies by creating clonal cell lines of MDA-MB-231 cells in which we artificially up-regulated the expression of exogenous hSP56. For this purpose we used a previously constructed mammalian expression vector which contains the entire cDNA coding sequence for hSP56 and was used successfully in the case of the PC-3 cell line. The expression of hSP56 in this vector is regulated by the strong human cytomegalovirus (CMV) immediate-early promoter. We transfected the vector into MDA-MB-231 cells and stably transfected clones were successfully selected using the limiting dilution method. Western blot analysis of hSP56 expression in the obtained clonal cell line MDA-MB-231/hSP56 confirmed that these cells express very high amounts of hSP56 protein (Fig. 10).

Next we wanted to determine if the effect of SeMet on the growth of MDA-MB-231 has been altered with the introduction of hSP56. The results show that the growth rate of this cell line in the presence of SeMet is markedly lower than that of the wild-type MDA-MB-231 cells (Fig. 11). As in the case of MCF-7 and T47-D cells the growth inhibitory action of SeMet was time- and concentration-dependent, but its effect was even more pronounced in the case of the MDA-MB-231/hSP56. This results are in accordance with our hypothesis, strongly suggesting that hSP56 is indeed involved in the growth inhibitory actions of SeMet in breast cancer cells.

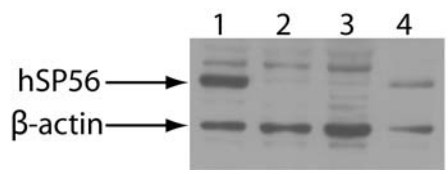

Figure 10. **Western blot analysis of hSP56 protein expression in MDA-MB-231/hSP56 cells.** Very high levels of hSP56 protein were detected in the clonal cell line MDA-MB-231/hSP56 (lane 1). hSP56 expressed in LNCaP cells (lane 4) was used as a positive control. Two other clonal cell lines of transfected MDA-MB-231 cells that did not express hSP56 are shown in lanes 2 and 3.

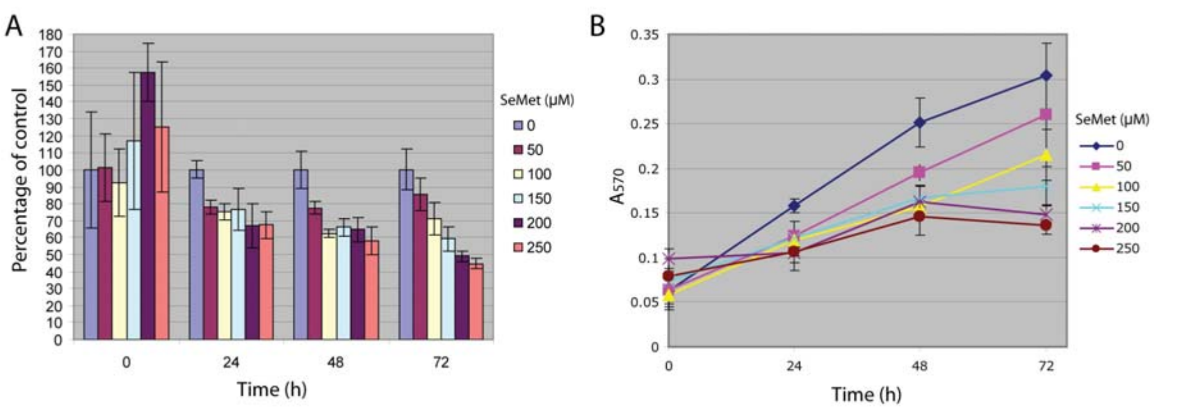

Figure 11. **SeMet displays a potent growth inhibitory action on MDA-MB-231/hSP56 cells.** The growth rates of MDA-MB-231/hSP56 cells in the presence of different concentrations of SeMet are presented as percentages of non-treated control (A) and as growth curves obtained from the measurements of absorbance at 570 nm (B). MDA-MB-231/hSP56, cells which express very high levels of hSP56, displayed significantly attenuated growth rates in the presence of SeMet and the number of cells treated with 250 μM SeMet for 72 h decreased to about 45%.

In order to establish stable down-regulation of endogenous hSP56 in breast cancer cell lines we decided to construct an hSP56 targeted short hairpin RNA (shRNA) expressing vector which can be stably integrated into the genome and can continuously produce shRNA molecules and trigger the RNAi response (6, 7). The 21 nt siRNA target sequence on hSP56 cDNA 5'-AAGGGCAATGGCAAAGGGGGT-3' (nucleotides 513-533 from NM_003944, NCBI) was chosen on the basis of the previous successful experiments in our lab, in which we down-regulated hSP56 in LNCaP prostate cancer cells using chemically synthesized siRNA oligonucleotides. The 21 nt target sequence served as a basis for the design of two complementary 55-mer shRNA template oligonucleotides that were synthesized, annealed and ligated into the linearized p*Silencer* 4.1-CMV neo expression vector (Ambion). A positive control plasmid was prepared in a parallel ligation reaction using the supplied GAPDH control insert. As part of our ongoing studies the obtained shRNA expression vectors, p*Silencer*-hSP56 and pSilencer-GAPDH, as well as the supplied negative control plasmid (encoding an shRNA whose sequence is not found in the mouse, human, or rat genome databases), will be transiently and stably transfected into hSP56 expressing cells and down-regulation of hSP56 will be analysed by western blot and RT-PCR. This will be followed by determination of the growth rates of the transfected breast cancer cell lines in the presence of SeMet. Additionally, using the optimal transfection conditions determined in the process of preparation of MDA-MB-231/hSP56 cells, we have successfully transfected the hSP56 mammalian expression vector into Hs578T cells (Fig. 3) and the selection of clonal cell lines is under way.

KEY RESEARCH ACCOMPLISHMENTS:

- hSP56 is expressed at high levels in the weakly invasive breast cancer cell lines MCF-7, SK-BR-3 and T-47D.

- hSP56 is undetectable in the highly invasive and tumorigenic MDA-MB-231 and Hs578T breast cancer cells.

- Evidence of a significant growth inhibitory activity of selenium compounds in the hSP56-expressing and weakly invasive breast cancer cell lines MCF-7 and T-47D.

- Evidence that selenomethionine displays a weak antiproliferative effect in the hSP56-deficient and highly invasive MDA-MB-231 and Hs578T cell lines.

- Successful transfection of mammalian expression vector containing hSP56 cDNA into MDA-MB-231 and Hs578T human breast cancer cells.

- Selection of stably transfected MDA-MB-231 cells that express hSP56 protein and isolation of clones from single cell colonies.

REPORTABLE OUTCOMES

- Applied for funding from NIH
- Manuscript describing above results completed and submitted
- Second manuscript in preparation

Sytkowski, AJ. Role of Human Selenium Binding Protein (hSP56) in the Pathobiology of Breast Cancer. Presented at 2005 Era of Hope Meeting, June 8-11, 2005, Philadelphia, PA

CONCLUSIONS

We have performed a basic characterization of hSP56 expression in selected breast cancer cell lines and determined their growth rates in the presence of selenomethionine, thus setting the grounds for our future studies. Most importantly, the potent antiproliferative activity of SeMet on MDA-MB-231/hSP56 cells, but not on MDA-MB-231 cells, is in strong agreement with our hypothesis that hSP56 mediates the growth inhibitory action of selenium compounds. The use of breast cancer cell lines with different tumorigenic properties and different hSP56 expression profiles is necessary in our studies of the function of hSP56 in breast cancer. Therefore, the prepared MDA-MB-231/hSP56 cells, as well as our ongoing work with Hs578T/hSP56 cells and shRNA downregulation of hSP56 will be valuable tools in our future studies. With these gain-of-function and loss-of-function studies we expect to gain new insights into the role of hSP56 in mediating the anti-cancer action of selenium in breast cancer.

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